MASTER COPY KEEP THIS COPY FOR REPRODUCTION PURPOSES

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	02/17/95	Final 06/01/91 - 11/30/94

4. TITLE AND SUBTITLE
Cloning of cDNA for two glutamate receptor proteins

5. FUNDING NUMBERS
DAAL03-91-G-0167

6. AUTHOR(S)
Elias K. Michaelis, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Kansas
Dept. of Pharmacology and Toxicology
and The Center for Biomedical Research
Lawrence, KS 66047

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P. O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
ARO 28669.1-LS

11. SUPPLEMENTARY NOTES
The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The studies described in the original proposal to the ARO were focused on the use of the immunochemical tools developed in these laboratories to identify the glutamate and CPP-binding protein cDNA's and clone these two cDNA's. The aims of these studies included the sequencing of the cloned cDNA's, the expression of the proteins in various cell systems such as COS and CV-1 cells, and measurement of ligand binding or ion channel properties of these receptor-related proteins. It was anticipated that following the successful completion of these studies, the cDNA libraries would be screened again in order to identify either homologous clones or clones for some of the other subunits of the complex of proteins isolated in these laboratories. This complex of proteins was proposed to form a glutamate/N-methyl-D-asparate (NMDA) receptor-ion channel. Part of the work proposed was focused on the documentation of such receptor activity being associated with the isolated complex of the proteins. The results described in this final report indicate that all of the goals of the proposed research were completed and that additional findings were documented that strongly support the hypothesis that the isolated proteins form a type of glutamate/NMDA receptor.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Membrane receptors; nervous system receptors; glutamate and NMDA receptors; organophosphate target receptor; protein isolation; cDNA cloning.	6
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

TITLE:
CLONING OF cDNA FOR TWO GLUTAMATE RECEPTOR PROTEINS

FINAL REPORT

AUTHOR OF REPORT
ELIAS K. MICHAELIS, PRINCIPAL INVESTIGATOR

DATE:
FEBRUARY 17, 1995

U.S. ARMY RESEARCH OFFICE

CONTRACT OR GRANT NUMBER:
DAAL03-91-G-0167

INSTITUTION:

THE UNIVERSITY OF KANSAS
DEPT. OF PHARMACOLOGY AND TOXICOLOGY
AND THE CENTER FOR BIOMEDICAL RESEARCH
UNIVERSITY OF KANSAS
LAWRENCE, KS 66047

A. STATEMENT OF THE PROBLEM STUDIED

Over the past three years our research efforts have been focused on the isolation from brain neuronal membranes of a complex of proteins that have the characteristics of a glutamate/NMDA receptor, the characterization of ligand binding sites associated with this complex and the analysis of the ion channel properties of the complex upon reconstitution in planar lipid bilayer membranes, and the cloning of theglutamate-binding, CPP-binding, and glycine-binding proteins of this complex.

B. SUMMARY OF THE MOST IMPORTANT RESULTS

1. Isolation of synaptic membrane proteins that are associated with an NMDA receptor-like function. A protein fraction eluted from an L-glutamate-ReactiGel matrix by introducing 5 mM NMDA into the elution buffer contains proteins with estimated molecular sizes based on migration in SDS-PAGE that are equal to 70, 62, 43 and 41 (doublet), and 36 and 31 (doublet) kDa (Kumar *et al.*, 1991; 1994). These proteins represent a greater than two-thousandfold enrichment of NMDA-sensitive L-[^{3}H]glutamate and MK-801-sensitive [^{3}H]TCP binding entities when compared with brain homogenates. The strychnine-insensitive [^{3}H]glycine binding activity in the NMDA-eluted protein fractions is 218 ± 95 pmol/mg (95 nM glycine, n=5 determinations) and that for [^{3}H]CPP binding is 100 ± 25 pmol/mg protein (100 nM CPP, n=6 determinations). These observations are indicative of the co-purification and enrichment of four recognition sites of NMDA receptors in this protein fraction. The estimated equilibrium dissociation constant (K_D) for L-glutamate binding to the agonist recognition sites in the complex is 99 nM and the maximum binding capacity (B_{max}) for these sites is 1.22 nmol/mg protein. The K_i for NMDA displacement of L-[^{3}H]glutamate bound to this protein complex is 490 nM. The estimated K_D for [^{3}H]TCP binding to the complex is 73 nM and the B_{max} is 1.5 nmol/mg protein. L-Glutamate and glycine added at a concentration of 5 μM and spermidine at 1 mM to the assay medium increased both the observed association rate constant (k_{obs}) and the B_{eq} for the binding of [^{3}H]TCP to the purified proteins. These observations are indicative of the presence of an apparently intact NMDA receptor-ion channel complex in these protein preparations in which the agonist, co-activator, and polyamine binding sites interact with the ion channel sites labeled by TCP. This complex has been purified further through chromatography on Sephacryl S-400 HR and sucrose density sedimentation to a final enrichment of NMDA receptor ligand-binding sites that is greater than 6,000-fold of those observed in whole brain homogenates (Kumar et al., 1994).

2. Reconstitution of the complex into planar lipid bilayer membranes. The possible formation of ion channels by the isolated complex of proteins has been examined in reconstitution studies in which both ion flux into liposomes (Ly and Michaelis, 1991) and ion channel conductance across planar lipid bilayers (Minami *et al.*, 1992; Michaelis et al., 1992) were measured. The Gaussian fits to all events collected from 6 reconstitution experiments reveal one major conductance state of 48 pS and two less frequent conductance states of 23 and 71 pS. The reversal potential for all three states of glutamate-induced openings of ion channels is approximately 0 mV. The slope conductances for the three types of channel open states were obtained from linear I-V relationships and are estimated to be 23.1 ± 0.7 pS, 46.8 ± 1.0 pS, and 74.3 ± 1.4 pS. The NMDA-activated conductance of these channels is blocked by the introduction into the "trans" chamber of the channel blocker MK-801 or by the co-introduction of two open-ion channel blockers, MK-801 and ketamine. Treatment of bilayers with the anti-70 kDa glutamate-binding protein antibody (IgG fraction, see below) introduced to the "trans" side (extracellular face of membrane) causes a decrease in the amplitude of channel events and in the total current measured over 50 s periods. Complete inhibition of all glutamate-activated currents was also achieved by the introduction into the "trans" face of the bilayer of a well characterized and highly specific monoclonal antibody to the 70 kDa glutamate binding protein. This antibody was used previously to screen cDNA libraries and clone the cDNA for the glutamate binding protein (Kumar *et al.*, 1991) and to label neurons and monitor the expression of this protein in neurons (Mattson *et al.*, 1991; 1993).

3. Isolation of subunits of a glutamate/NMDA receptor complex and immunochemical characterization of the isolated proteins. The purification of 63–71 kDa glutamate-binding proteins from rat brain synaptic membranes was previously reported from this laboratory (Chen *et al.*, 1988; Wang *et al.*, 1992). Antibodies raised against the purified glutamate-binding protein (Chen *et al.*, 1988; Eaton *et al.*, 1990) label a 63–71 kDa protein band in synaptic membranes (Chen *et al.*, 1988; Eaton *et al.*, 1990, Wang *et al.*, 1992), in protein fractions eluted by NMDA from glutamate ReactiGel affinity matrices (Kumar *et al.*, 1991), and in the fractions purified through chromatography on Sephacryl S-400 HR (Kumar et al., 1994).

A 54–58 kDa protein was first purified in our laboratories and characterized as a CPP-, 2-AP5-, 2-AP7-, and CGP 39653-binding protein (Cunningham and Michaelis, 1990; Eggeman *et al.*, 1993). Antibodies raised against the 54–58 kDa CPP and CGP 39653-binding protein most often labeled a protein species of 79-83 kDa molecular size in synaptic membranes (Eggeman *et al.*, 1993). The anti-54 kDa antibodies also immunoextract 62% of the [^{3}H]CGP 39653 binding sites from solubilized synaptic membrane proteins (Eggeman *et al.*, 1993).

In order to probe the structure and function of the other two putative subunits of a glutamate/NMDA receptor complex, *i.e.*, the 41–43 and 31–36 kDa proteins, antibodies were raised against these proteins following purification through affinity chromatography on glutamate-ReactiGel columns and electroelution of the specific protein bands from SDS-PAGE. Also, affinity chromatography procedures were developed for the purification of a glycine-binding protein that we believe is part of the complex identified above. Purification of a protein of ~60 kDa to homogeneity has been achieved by this technique, and this protein binds [^{3}H]glycine with a stoichiometry of 1 mol glycine per mol of protein.

4. Cloning of the CPP-binding and glycine-binding protein cDNAs and studies of the expression of these proteins in brain. The cloning of the cDNA for the 71 kDa glutamate-binding protein was achieved in 1991 (Kumar *et al.*, 1991). The structure of this protein bears no significant homology to the structure of either NMDAR1 or NMDAR2 or the kainate/AMPA receptors. *E. coli* transformed with the cDNA for the glutamate binding protein express the protein in a state that binds L-glutamic acid with the same affinity as that of the protein purified from brain (Kumar *et al.*, 1991). Site-directed mutations were introduced in the expressed protein to probe the regions that contribute predominantly to the expression of ligand binding activity (Hong *et al.*, unpublished observations). A region near the C-terminal of the protein has been identified which plays a dominant role in the expression of glutamate binding activity. Mutants which express a protein that has the whole N-terminal region up to the putative transmembrane domain #4 removed, exhibit higher levels of binding activity than the mutants in which 14 amino acids near the C-terminal region were deleted. The change in ligand binding characteristics of the mutated proteins in this critical 14 amino acid region is a decrease in both K_D and B_{max} for L-glutamic acid binding to the protein. These observations confirm the likely extramembranous position of the C-terminal of the protein and indicate that expression of this protein in bacterial cells provides a useful model for the study of ligand binding selectivity.

The antibodies raised against the previously purified 54 kDa fragment of the CPP or CGP 39653-binding protein have also been used in library screening and a 2.8 kb cDNA was cloned, which upon expression in bacteria leads to the production of an 83 kDa protein which binds both [^{3}H]CPP and CGP 39653 (Johnson *et al.*, 1992). Preliminary studies indicate that this cloned and expressed protein has unique NMDA receptor sites that are sensitive to organophosphate inhibition (Johnson and Michaelis, unpublished observations). The sequence of the cloned cDNA already obtained is not homologous to other NMDA receptor proteins. A larger cDNA clone of 3.8 kb for the CPP-binding protein was also isolated and the sequencing of this clone is now nearly complete. Both the 3.8 and 2.8 kb clones label brain mRNA that has an estimated size of 3.8 kb, which is indicative that the 3.8 kb clone represents the complete message for this protein. Both the glutamate-binding and the CPP-binding proteins have recently been stably expressed in mammalian cells (green monkey kidney CV-1 cells) using the stable episomal expression of a vector that includes the Epstein Barr Virus EBNA1 and Ori regions. Cells

stably transformed were selected through expression of antibiotic resistance genes for G418 and hygromycin. Each of the cell lines that are stably expressing these proteins showed immunochemically recognizable proteins of 66 kDa for the glutamate-binding protein and 83 kDa for the CPP-binding protein.

In the most recent studies, cerebellar libraries were screened with the anti-43 kDa antibodies and eleven clones were identified as expressing an immunoreactive protein. The largest insert in one of these clones is 1.8 kb. The sequence for this cDNA was completed and it is a unique sequence with only 20% homology to the glutamate-binding protein (NMDARA1) but no homology to either NMDAR1 or R2 proteins. Northern blot analyses of rat brain RNAs performed using the cloned cDNA reveal a 1.8 kb brain mRNA that is strongly labeled. This indicates that the 1.8 kb cDNA insert is the complete message for this protein. This protein and its cDNA are referred to as NMDARA3. The transformation of *E. coli* with the cDNA for the NMDARA3 leads to the expression of strychnine-insensitive but 5,7-dichlorokynurenic acid-sensitive [^{3}H]glycine binding entities which also contain [^{3}H]TCP binding sites.

One of the key observations for both the NMDARA3 purified from brain synaptic membranes as well as the protein expressed from the cloned cDNA is that the TCP binding activity of the protein is very strongly enhanced by L-glutamate and glycine. These observations are a clear indication that this protein is indeed a component of an NMDA receptor in brain.

C. LIST OF PUBLICATIONS

Ly, A.M. and Michaelis, E.K. Solubilization, partial purification, and reconstitution of glutamate and N-methyl-D-aspartate-activated cation channels from brain synaptic membranes. Biochemistry, 1991, 30, 4307-4316.

Kumar, K.N., Eggeman, K.T., Adams, J.L., and Michaelis, E.K. Hydrodynamic properties of the purified glutamate-binding protein subunit of the N-Methyl-D-Aspartate receptor. Journal Biological Chemistry, 1991, 266, 14947-14952.

Mattson, M.P., Wong, H., and Michaelis, E.K. Developmental expression, compartmentalization, and possible role in excitotoxcity of the putative glutamate receptor protein in cultured hippocampal neurons. Brain Research, 1991, 565, 94-108.

Kumar, K.N, Tilakaratne, N., Johnson, P.S., Allen, A.E. and Michaelis, E.K. Isolation of the NMDA receptor complex and cloning of cDNA for the glutamate-binding subunit. Nature, 1991, 354, 70-73.

Minami, H., Sugawara, M., Odashima, K., Umezawa, Y., Uto, M., Kuwana, T. and Michaelis, E.K. Ion channel sensors for glutamic acid. Analytical Chemistry, 1991, 63, 2787-2795.

Minami, H., Uto, M., Sugawara, M., Odashima, K., Umezawa, Y., Michaelis, E.K. and Kuwana, T. An evaluation of signal amplification by the ion channel sensor based on a glutamate receptor ion channel protein. Analytical Sciences, 1991, 7, 1675-1676.

Michaelis, E.K. and Michaelis, M.L. Molecular aspects of glutamate receptors and sodium-calcium exchange carriers in mammalian brain: Implications for neuronal development and degeneration. Neurochemical Research, 1992, 17, 29-34.

Wang, H., Kumar, K.N. and Michaelis, E.K. Isolation of glutamate-binding proteins from rat and bovine brain synaptic membranes and immunochemical characterization. Neuroscience, 1992, 46, 793-806.

Balazs, R., Resink, A., Hack, N., Van der Valk, J.B.F., Kumar, K.N. and Michaelis, E.K. NMDA treatment and K^+-induced depolarization selectively promote the expression of an NMDA-preferring class of the ionotropic glutamate receptors in cerebellar granule neurones. Neuroscience Letters, 1992, 137, 109-113.

Michaelis, E.K., Michaelis, M.L., Kumar, K.N., Tilakaratne, N., Joseph, D.B., Johnson, P.S., Babcock, K.K., Schowen, R.L., Minami, H., Sugawara, M., Odashima, K., and Umezawa, Y. Purification, reconstitution and cloning of an NMDA receptor-ion channel complex from rat brain synaptic membranes: Implications for neurobiological changes in alcoholism. Annals N.Y. Acad. Sci., 1992, 654, 7-18.

Johnson, P.S. and Michaelis, E.K. Characterization of organophosphate interactions at N-methyl-D-aspartate receptors in brain synaptic membranes. Mol. Pharmacol., 1992, 41, 750-756.

Eggeman, K.T., Pal, R., Walsh, J., Kumar, K.N., and Michaelis, E.K. Immunochemical and immunohistochemical characterization of a synaptic membrane protein that binds the competitive antagonists of NMDA receptors. Neuroscience Letters, 1993, 158, 173-176.

Mattson, M.P., Kumar, K.N., Wang, H., Cheng, B., and Michaelis, E.K. Basic FGF regulates the expression of a functional 71 kDa NMDA receptor protein that mediates calcium influx and neurotoxicity in hippocampal neurons. Journal of Neuroscience, 1993, 13, 4575-4588.

Collins, C., Duff, C., Montal, M., Duncan, A.M.V., Planelis-Cases, R., Norremolle, A., Michaelis, E.K., Worton, R., and Hayden, M.R. Mapping of the human NMDA receptor subunit (NMDAR1) and the proposed NMDA receptor glutamate-binding subunit (NMDARA-1) to chromosome 9q34.3 and chromosome 8, respectively. Genomics 17, 1993, 17, 237-239.

Kumar, K. N., Babcock, K. K., Johnson, P. S., Chen, X., Eggeman, K. T., and Michaelis, E. K. Purification and pharmacological and immunochemical characterization of synaptic membrane proteins with ligand-binding properties of N-methyl-D-aspartate receptors. Journal of Biological Chemistry 269, 1994, 27384-27393.

Xia, Y., Ragan, R. E., Ching Seah, E. E., Michaelis, M. L., and Michaelis, E. K. Developmental expression of N-methyl-D-aspartate (NMDA)-induced neurotoxicity, NMDA receptor function, and the NMDAR1 and glutamate binding protein subunits in cerebellar granule cells in primary cultures. Neurochemical Research, 1995, in press.

Michaelis, E.K., Kumar, K.N., Tilakaratne, N., Johnson, P.S., Allen, A.E., Aistrup, G., and Schowen, R. Purification and reconstitution of an NMDA receptor-ion channel complex and cloning of a cDNA for the glutamate binding subunit. In Excitatory Amino Acids- FIDIA Research Foundation Symposium Series, Vol. 9, 1992, R.P. Simon, Ed., Thieme Medical Publishers, Inc., New York, pp. 23-27.

Michaelis, E.K. Two different families of NMDA receptors: Physiological function and role in neuronal development and degeneration. In Advances in Experimental Medicine and Biology, S. Grisolia, V. Felipo, Eds., Plenum Press, New York, 1994, 341, 119-138.

Michaelis, E.K. Two different families of NMDA receptors: Physiological function and role in neuronal development and degeneration. In Advances in Experimental Medicine and Biology, S. Grisolia, V. Felipo, Eds., Plenum Press, New York, 1994, 341, 119-138.

Michaelis, E.K. Glutamate Receptors. In Encyclopedia of Neuroscience, G. Adelman and B. Smit, 1995, invited contribution.

SCIENTIFIC PERSONNEL SUPPORTED BY THIS PROJECT AND DEGREES AWARDED DURING THIS REPORTING PERIOD:

Dr. E.K. Michaelis, principal investigator
Dr. M.L. Michaelis, co-principal investigator
Dr. K.N. Kumar, assistant scientist
Dr. M. Ahmad, post-doctoral associate
Dr. Shabana Islam, post-doctoral associate
P. S. Johnson, graduate student
K. T. Eggeman, graduate student
O. Hong, graduate student
A. Allen, graduate student
G. Aistrup, graduate student
O. Jing, research assistant
A. Rigel, research assistant

Degrees:

K. T. Eggeman, Ph.D.
O. Hong, Ph.D.
P. Johnson, Ph.D.